



First DNA barcoding and phylogenetics of wild *Betta edithae* (Anabantiformes: Osphronemidae) from Belitung Island, Indonesia

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Abstract. *Betta edithae* is a freshwater species only met in the Greater Sunda Islands region in Indonesia and spread in Borneo, Sumatra, Bangka Belitung Island and the Riau Archipelago. This study was intended to record for the first time the genetics of *B. edithae* and update the nucleotide composition of *Betta edithae* from the island of Belitung. The 25 studied specimens were obtained in the swamp forest stream of Dukong Village, Simpang Pesak District, East Belitung Regency, Belitung Island, Indonesia from 29th to 30th of June 2023. These specimens exhibited a total length of 2-3 cm. The study presents the pioneering documentation of *B. edithae* DNA barcoding, focusing on the Cytochrome C oxidase Subunit I (COI) gene from Belitung Island. DNA-Barcode of *Betta edithae* from Belitung Island has a base-pair length of 686 bp and is closed to the *B. edithae* from Kalimantan, with a level of similarity of about 91%, a query coverage of 95% and a genetic distance of around 0.10. The recorded DNA Barcode of *B. edithae* has been officially registered in the Genbank under the accession code OR166098. DNA barcoding is an advanced and expedient method for precise species identification and discovery, revolutionizing the field of taxonomy. Phylogenetic analysis revealed that *B. edithae* forms a distinct clade alongside the *Betta* genus, shedding light on its evolutionary relationships and genetic affinities.

Key Words: biodiversity, biogeography, endemic betta, phylogenetic analysis.

Introduction. In terms of fish variety, Indonesia is a mega-biodiversity nation; there are now 8.500 fish species that have been categorized based on habitat characteristics (such as salty, brackish, and freshwater) (Ndobe et al 2023; Hasan et al 2023; Gani et al 2021; Syarif et al 2019; Aisyah & Syarif 2018; Syarif et al 2016). At least 1,266 species, including endemic, native, introduced, and reintroduced species, have been identified in Indonesian inland waters (Robin et al 2023a; Kurniawan et al 2022; Serdiati et al 2020; Insani et al 2020; Ihwan et al 2020; Syarif & Prasetyono 2019). As a group of freshwater species, *Betta* (Bleeker 1850) is the most diversified genus within the Osphronemidae family. In South-East Asia, there are around 65 different species of *Betta* (Schindler & Linke 2013; Tan et al 2009); globally, there are roughly 74 different species of *Betta* that are split into several groups of species. Tan & Ng (2005a) described a total of 14 groups made up of various species in 2005. *Betta edithae* is the only species in the Edithae group. Depending on the type of parental care, the genus is traditionally divided into mouth-growing and bubble-nesting species (Tan & Ng 2005a; Tan et al 2013).

B. edithae is a species from the Perciformes ordo and Osphronemidae family. This species is known only from the Greater Sunda Islands region in Indonesia. Like many betta species, *B. edithae* is able to adapt and survive in extreme environmental conditions such as the highly acidic blackwater (pH 3-5) of peat swamp forests (Tan & Ng 2005a). Betta species also tend to restrict their habitat to one or more drainage systems separated by various obstacles such as mountains and major rivers. As an endemic fish to Indonesia, *B. edithae* has a fairly wide distribution in the western region of Indonesia starting from Kalimantan Timur, Kalimantan Selatan, Kalimantan Tengah, Kalimantan Barat, Sumatra Selatan, Banka, Biliton and Pulau Bintan (of the Riau Archipelago) (Tan & Ng 2005b). The

last record of this species is also known to be on the Indonesian Belitung Island (Syarif et al 2020).

Belitung Island is one of the remote islands of Indonesia and is in the Greater Sunda Islands region in Indonesia. In addition, Belitung Island is up to 700 km southeast of the nearest locality in Peninsular Malaysia, about 250 km southeast of the nearest locality in Bangka Island, about 600 km southeast of the nearest locality in Sumatra, and about 500 km south-west from the nearest locality in Borneo (Hasan & South 2023). Due to its location in Sundaland and the presence of numerous once-connected ancient rivers, Belitung Island boasts a rich biodiversity and a wide range of indigenous species. Several species of wild betta fish, which are commonly known as natural betta fish (Tapalak/Tempalak), are also widely distributed in the Belitung Islands, due to their characteristics of peat swamp forest with highly acidic blackwater (pH 3-5) (Syarif et al 2021).

Morphologically, *B. edithae* from Belitung Island has been confirmed (Syarif et al 2020; Syarif et al 2021). To ensure the effectiveness of domestication and conservation initiatives, this species must be validated. A genetic study approach is required to determine species certainty. In this study, we present the molecular identification of the *B. edithae* from Belitung Island, Indonesia, based on the COI gene. The COI gene has been chosen as a standard tool for molecular taxonomy and identification worldwide (Bingpeng et al 2018), and can be relied upon as a basis for differentiation between animals (Liu et al 2020). The use of the COI gene as a species identification tool has been successfully carried out for the identification of freshwater fish in Indonesia (Insani et al 2023; Valen et al 2022c). The DNA barcoding will be uploaded to the Genbank NCBI database as a standard for recognizing wild *B. edithae* specie. This research will support information about the rapid species identification, understanding of biodiversity, increasing species genetic diversity, life history of species and, in the long term, will support conservation actions and development.

Material and Method

Sampling site and fish samples collection and water quality. The study was conducted from 29th to 30th of June 2023 in the Swamp Forest Stream of Dukong Village, Simpang Pesak District, East Belitung Regency, Belitung Island, Indonesia. Samples were collected using environmentally acceptable fishing equipment, i.e. traps with dimensions of 90 x 90 x 40 cm³, made of wire and mesh. A total of two trap units were installed at two different depths, specifically 0.5 m and 1.0 m from the river surface. The fish caught in the traps were examined daily for a week after being set. From both traps, all of the fish that had been caught were gathered and taken to the lab for the next research stage. We also conduct in-situ studies of water quality, including temperature, pH, and dissolved oxygen, as part of the study of the habitat of the wild *B. edithae*.

Preserve fish and morphological analysis. A total of 25 specimens of *B. edithae* were caught using the fish trap during a week of trapping. The remaining 23 were kept as livestock at the Fish Reproduction Laboratory, Bangka Belitung University, Indonesia for domestication. However, at least 2 wild *B. edithae* specimens were dissected for research (Metcalf & Craig 2011). The right body's pectoral fin of one specimen was removed for DNA extraction and the left side was used for morphometric data collection. The specimen was preserved in a 96% alcohol solution (Valen et al 2022a). The other specimen was preserved in a 7% formalin solution (Valen et al 2020) and deposited at the zoology laboratory, at Bangka Belitung University. Diagnostic morphological characters of the specimen were analyzed following Tan & Ng (2005a) and identified as *B. edithae* with specimens measuring 2-3 cm. The determined meristic characters include dorsal fin rays, ventral fin rays, pectoral fin rays, and anal fin rays. Other morphological characteristics of the specimen were measured using digital calipers to the nearest 0.1 mm.

DNA extraction and amplification. The process of extracting and amplifying DNA was carried out between the dates of July 5th and July 10th, 2023. DNA isolation was conducted

using the Nexpro™ DNA kit at the Basic Biology Laboratory, University of Bangka Belitung, with the FISH-F2 and FISH-R2 primers (Ward et al 2005). The PCR process refers to the procedure of Aisyah et al (2021) by using 2% forward and reverse primers, 50% PCR mix of Nexpro™ Master mix, 36% ddH₂O, and 10% DNA template. The reaction mixture was then amplified using a BioRad type T100™. PCR cycling parameters included an initial pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 54°C, extension at 72°C for 10 seconds (35 cycles), and final extension at 72°C for 10 minutes. The PCR results were then visualized in 1% agarose gel via electrophoresis by staining with Nucleic Acid Gel Stain (GelRed®) (Robin et al 2023b; Hasan et al 2021; Valen et al 2021). The agarose was a mixture with 5 mL of TAE buffer and 45 mL of distilled water, and 8 µL of red gel stain was added to the solution. 5 µL of DNA sample was homogenized with 1 µL of loading dye and added to the agarose gel wells. The positive sample (sparkling DNA bands) was then processed for DNA reading (sequencing) using the Sanger dideoxy method at PT Genetics Science Jakarta.

Data analysis. Additional sequences were acquired using BLAST searches of the Genbank database, and they were used to identify specimens. We registered the DNA Barcode of the sequence to the Genbank (National Center for Biotechnology Information 1988) (<https://www.ncbi.nlm.nih.gov/genbank/>) with the accession number OQ281707. The evolutionary history of the species was determined by using the Neighbor-Joining method (Saitou & Nei 1987) with the bootstrap test (1,000 replicates) (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al 2004) in MEGAX software (Kumar et al 2018).

Results

DNA barcoding. DNA-barcode of *Betta edithae* from Belitung Island was successfully sequenced with a base-pair length of 686 bp (Table 1) using Fish_F2 and Fish_R2 primers (Ward et al 2005).

Table 1

DNA barcoding of *Betta edithae* from Belitung Island, Indonesia

DNA barcoding of <i>B. edithae</i>
GGAATGGTCGGTACCGCTTTAAGTCTACTCATTTCGAGCAGAGTTAAGTCAACCAGGAGCTCTTCT TGGAAACGACCAGATTTATAATGTAATTGTTACGGCACACGCTTTTGTATAATCTTTTTATGGTT ATACCAGTTATAATCGGCGGCTTCGGGAATTGACTCGTCCCTCTTATAATTGGAGCACCAGATATA GCCTTTCCCGAATAAATAACATAAGCTTTTGACTTTTACCCssssssssssCCATCCTTTCTTCTGCT ATTAACATCCTCTGGTGTAGAAGCCGGAGCAGGGACCGGTTGGACTGTATATCCACCCCTGGCCA GCAACTTGGCACACGCAGGTGCTTCTGTAGACTTAACAATTTTTCACTCCACCTAGCAGGTGTGT CATCTATTTTAGGGGCTATTAATTTATCACCACAATTATTAACATGAAACCACCCGAGTCTCCA ATACCAAACACCGCTGTTTGTGAGCCGCTTAGTAACAGCAGTATTACTTCTTTATCGCTTCCT GTCCTAGCCGCCGGGATTACAATGCTTCTAACAGATCGAAACCTAAACACAACCTTTCTTCGACCCT GCAGGGGGCGGAGACCCAATCCTATATCAACACTTATTCTGATTCTTTGGCCACCAGAGAAAATC TCTAAAA

Molecular identification. The DNA Barcoding of the sequence from the Belitung Island was analyzed and compared to the NCBI GenBank (National Center for Biotechnology Information 1988) via BLAST (Basic Local Alignment Search Tool-nucleotide) method (<https://blast.ncbi.nlm.nih.gov>) and the BOLD system, via the Identification Engine, to find out and analyze a sequence homology. We found that the sequence from Belitung Island is close to the *B. edithae*, with a level of similarity about 91% (Table 2).

Table 2

Species identification and similarity

Species outcome	Family	Accession ID	Query coverage (%)	Percent identity (%)
<i>Betta edithae</i>	Osphronemidae	KM485319.1	95	91.04
<i>Betta pi</i>	Osphronemidae	MK074103.1	99	88.69
<i>Betta hipposideros</i>	Osphronemidae	KM485457.1	95	89.15
<i>Betta chloroparinx</i>	Osphronemidae	KM485404.1	93	89.68
<i>Betta waseri</i>	Osphronemidae	KM485458.1	95	88.94
<i>Betta krataiois</i>	Osphronemidae	KM485406.1	95	88.84
<i>Betta fusca</i>	Osphronemidae	GQ911728.1	95	88.82
<i>Betta pugnax</i>	Osphronemidae	MW591014.1	95	87.85
<i>Betta pulchra</i>	Osphronemidae	KM485449.1	95	87.72

Classification. Based on Blast DNA applied to the DNA barcode of *B. edithae* from the swamp forest stream of Dukong Village, Simpang Pesak District, East Belitung Regency, Belitung Island, Indonesia, the sample was classified as follows:

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Osphronemidae

Genus: *Betta*

Species: *Betta edithae*

Local name: Ikan Tepalak/Tempalak

Identification and grouping of species into categories or classifications are very important to conclude on the results of species identification in a more complete manner. Classification was carried out based on cytochrome c oxidase sub unit I (COI) gene. The research results will form the basis for the species conservation and domestication process.

Water quality. The water quality values in the downstream, middle and upstream waters were different. In the downstream waters the temperature ranged from 27.8 to 30.2°C, the pH value ranges from 3.6 to 3.9, and the DO value ranged from 6.41 ppm to 7.81 ppm. In the middle streams of the water temperatures ranged from 27.9 to 30.2°C, pH values ranged from 4.9 to 5.6, and DO values ranging from 5.64-8.02 mg L⁻². In the upstream waters temperatures ranged from 26.8 to 30.2°C, pH values ranged from 4.0 to 4.9, and DO values ranging from 5.27 to 8.24 mg L⁻² (Table 3).

Table 3

Water quality in the swamp forest stream of Dukong Village, Belitung Island, Indonesia

Water quality parameters	Sampling sites		
	Upstream	Middlestream	Downstream
Temperature	26.8-30.2	27.9-30.2	27.8-30.2
pH	4.0-4.9	4.9-5.6	3.6-3.9
Dissolved oxygen	5.27-8.24	5.64-8.02	6.41-7.81
Water color	Black	Brown	Blackish Red

Discussion. The DNA barcoding can be used as a reference for species identification (Guo et al 2022). This information is very important to enrich the science, especially to understand the taxonomy (Tadmor-Levi 2022; Robin et al 2023c) and to improve knowledge in biotechnology. The DNA Barcode of *B. edithae* is Indonesia's first report and the first barcode registered in the Genbank. Based on the results of the analysis of BLAST DNA sequences from Belitung Island, it was found that the sequences from Belitung Island were similar to *B. edithae* from Kalimantan with the GenBank access code KM485319.1

with a similarity percentage of 91% and query coverage of 95%. Hebert et al (2003) stated that species with 97-100% similarity levels are identical, so that species differences above 3% based on COI genes lead to new species. However, based on the results of morphological (meristic and morphometric) analysis, this specimen belongs to the *B. edithae* species (Syarif et al 2020; Syarif et al 2021). Even said, when compared to other varieties of Betta, the sequence from Belitung is still more similar to *B. edithae* from Kalimantan. Tan et al (2005a) also grouped *B. edithae* into the *B. edithae* group, as the only species in this group. Therefore, there is little chance of misidentification because the sequence was taken from a specimen captured in Belitung, which definitely belongs to *B. edithae*, morphologically. According to our conjecture, the environmental stresses in Belitung have caused a shift in the nucleotide makeup of the *B. edithae* from Belitung, which has caused it to diverge significantly from its relatives in Kalimantan.

Belitung Island's freshwater resources are directly impacted by the open-air tin mining, which causes the waters to become acidic (pH approaches 3), with low quantities of dissolved oxygen and heavy metal contaminants (Kurniawan & Mustikasari 2021; Hashim et al 2018; Kurniawan et al 2020). Acidity, hypoxic water conditions and heavy metals or toxic metals contamination can be factors of serious extreme environmental stress for the life of organisms (Kurniawan & Mustikasari 2021; Thompson et al 2017; Igiri et al 2018). However, there are some fish that can live in extreme conditions, called extremophile fish. Fish classified as extremophiles are species that have adapted to this harsh environment (Kurniawan & Mustikasari 2021). Adaptation to this extreme environment occurs outside and inside the body of the fish, related to the nucleotide composition of a species, which leads to the process of evolution and produces new species.

The swamp forest stream of Dukong Village (Simpang Pesak District, East Belitung Regency, Belitung Island, Indonesia) exhibit high tannin content and low pH. The pH values range from 4.0 to 4.9 in the upstream waters, from 3.6 to 3.9 in the downstream waters, and from 4.9 to 5.6 in the middle streams. Wild Betta fish generally inhabit swamp habitats with a fairly low pH, of 4-5 (Tan & Ng 2005), while a normal pH for fish life is between 6.5-8.5. According to Amaral-Zettler (2013), habitats with an extreme pH are those that are either extremely acidic (pH 3) or extremely alkaline (pH>9) in nature.

The DNA barcode of *B. edithae* has a high average proportion of adenine and thymine, which is known as the A-T rich group, accounting for roughly 60% of the total nucleotides, according to the results of sequence analysis based on the COI gene from Belitung Island. The A-T hydrogen bond consists of 2 hydrogen bonds which are weaker than the G-C hydrogen bond which has 3 hydrogen bonds, so the possibility of species mutation is higher. The complete percentage of nucleotide composition of *B. edithae* was T (30.7%), C (25.4%), A (23.3.0%), and G (20.6%). Total DNA-barcode of *B. edithae* from Belitung Island is 686 bp. According to Guo et al (2022), COI gene sequences with more than 658 base pairs can be used as a standard for differentiating animals. Hebert et al (2003) stated that the mitochondrial DNA cytochrome oxidase subunit I (COI) gene has the capability to serve as a barcode of all animals. The sequence data from this research has also been submitted to the Genbank (National Center for Biotechnology Information 1988) (<https://www.ncbi.nlm.nih.gov/genbank/>) with Accession Number OR166098. In the future, this sequence will serve as a reference for species identification using DNA barcoding and COI gene markers (Insani et al 2023; Liu et al 2020). This information is very important to enrich science, especially to understand the taxonomy (Tadmor-Levi 2022) and improve knowledge in biotechnology. Registration of this sequence also aims to describe the eukaryotic biodiversity in support of the Barcode of Life (BOL) project. The genetic samples of species are also useful for understanding the population structure. The populations of several native and endemic fishes are declining on a global scale, due to the presence of invasive and non-native species (Valen et al 2022b; Hasan et al 2020).

In the current paper, the phylogenetic tree is also constructed by using the Neighbor-Joining method based on the Tamura-Nei model (Tamura et al 2004), with the MEGA X (Kumar et al 2018), with 1,000 replicates, to show the evolutionary tree of the species (Figure 1). The sequences of the Betta family (26 species and 14 groups) were taken from the Genbank to complete the phylogenetic tree based on the COI gene.

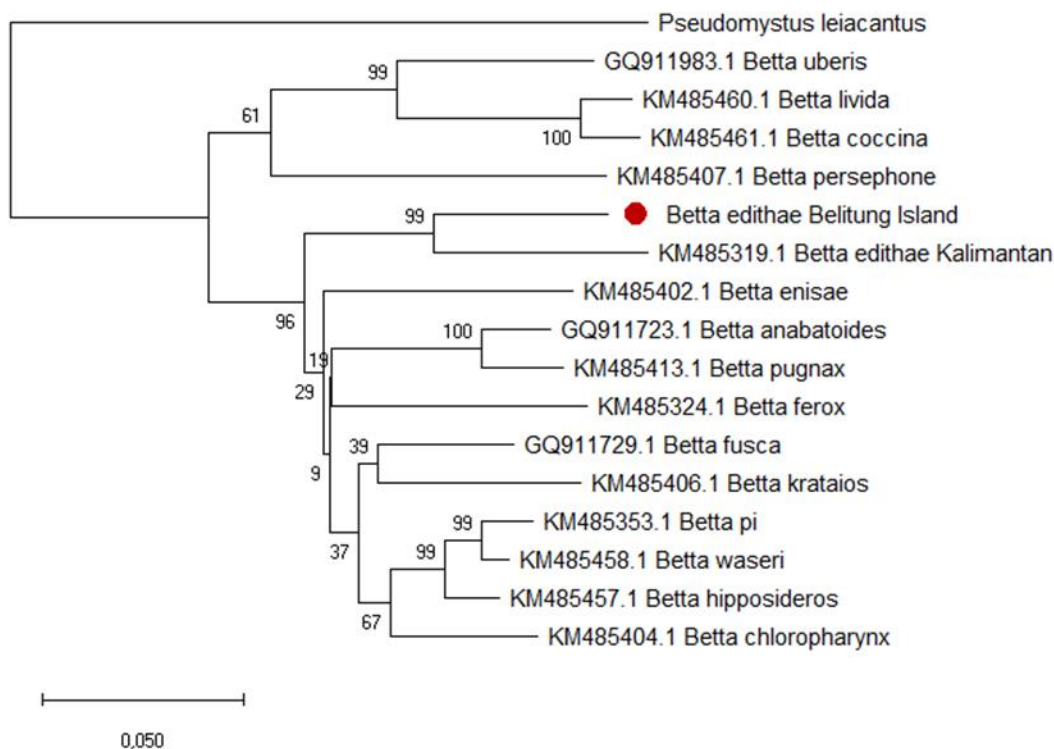


Figure 1. The evolutionary tree of *Betta* genus based on COI.

The phylogenetic tree shows that *B. edithae* from Belitung island is closed to *B. edithae* Kalimantan. As can be seen, the phylogenetic tree of *B. edithae* was clustered in a clade in the *Betta* genus and was close to *B. edithae* Kalimantan with a genetic distance of around 0.10 (Table 4).

Table 4
Estimates of evolutionary divergence between *Betta* genus based on COI gene

	1	2	3	4	5	6	7	8	9	10	11	12
1 <i>Betta edithae</i> Belitung												
2 <i>Betta anabatooides</i>	0,14											
3 <i>Betta fusca</i>	0,13	0,11										
4 <i>Betta uberis</i>	0,21	0,21	0,16									
5 <i>Betta edithae</i> Kalimantan	0,10	0,14	0,13	0,21								
6 <i>Betta ferox</i>	0,16	0,12	0,11	0,17	0,16							
7 <i>Betta pi</i>	0,12	0,11	0,08	0,17	0,14	0,11						
8 <i>Betta enisae</i>	0,15	0,13	0,12	0,19	0,15	0,13	0,12					
9 <i>Betta chloropharynx</i>	0,12	0,10	0,08	0,19	0,14	0,12	0,07	0,12				
10 <i>Betta krataios</i>	0,13	0,12	0,09	0,19	0,16	0,14	0,10	0,11	0,11			
11 <i>Betta persephone</i>	0,21	0,20	0,18	0,16	0,21	0,20	0,19	0,18	0,19	0,20		
12 <i>Betta pugnax</i>	0,14	0,04	0,11	0,21	0,14	0,12	0,12	0,12	0,10	0,12	0,20	
13 <i>Betta hipposideros</i>	0,12	0,11	0,06	0,15	0,14	0,10	0,04	0,12	0,06	0,10	0,18	0,11

B. edithae is a species of endemic fish from Indonesia, with distribution in the western region. Based on the COI gene, *B. edithae* from the Genebank was recorded from Kalimantan (Panijpan et al 2014). In this research, we recorded the genetics of *B. edithae* from Belitung Island based on the COI gene. Belitung Island is a remote island in the Greater Sunda Islands region in Indonesia. In addition, Belitung Island is up to about 500 km southwest of the nearest locality in Kalimantan. Due to its location in Sundaland, Kalimantan, and Borneo were once connected to the ancient rivers of Sundaland (Bennett et al 2016). Due to their historical connections and subsequent species migration, most of the species owned by these two places are the same. Different environmental stresses have an impact on how organisms change in terms of their physical characteristics and the order of their genetic code. Belitung Island has an extreme environmental pressure from the water quality, due to the negative effects of open-pit tin mining, which forces Belitung

species to adapt to extremes (Kusumah et al 2023). This is evidenced by the large genetic distance (0.10) between *B. edithae* from Belitung and Kalimantan. It can also be seen in the evolutionary tree, where *B. edithae* from Belitung and Kalimantan are evolutionarily close, coming from one ancestor who is closely related compared to other species in the same genus, but the evolutionary distance between them is starting to be significant. We speculate that *B. edithae* from Belitung Island is a cryptic species of the Edithae group.

In this study, we succeeded in reporting the first record of DNA Barcoding of *B. edithae* based on the COI gene from the Belitung Islands which is the first step in confirming the species and showing genetic differences between the *B. edithae* sequences from Belitung Island and sequences from Kalimantan Island. Furthermore, we suggest conducting further research on species identification based on the whole genome, using mitochondrial DNA, and a more in-depth morphological analysis to determine the species status of *B. edithae* from Belitung Island, because we believe these species are different but belong to the same group. In addition, studies regarding genetic diversity and population structure will also be supported by the DNA barcoding, especially for the selection of brood in domestication efforts and to determine the number of species' populations.

Conclusions. This study discovered and documented the first genetic *B. edithae* from the swamp forest stream of Dukong Village, Simpang Pesak District, East Belitung Regency, Belitung Island, Indonesia. Several other fish species were also found in the same habitat during the sampling. A 686 base-pair length sequence from DNA barcoding of *B. edithae* from Belitung Island was obtained. The nucleotide composition of the DNA barcode indicated a high content of adenine and thymine, categorizing the COI gene of this genus as A-T rich. The study confirmed that the COI gene accurately identifies species, as the sequence belonged to the *Betta* genus, consistently with morphology-based studies. The *B. edithae* 686 bp COI gene sequence satisfied the requirements for animal differentiation. The DNA barcode of *B. edithae* from Belitung Island was the first record for Belitung and the second record for Indonesia. The genetic information obtained from this research has significant implications for enriching scientific knowledge, especially in the fields of taxonomy and biotechnology. It contributes to the registration of eukaryotic biodiversity and supports the Barcode of Life project. The genetic samples collected in this study are valuable for understanding population structure, particularly in the context of declining populations of native and endemic fish species caused by the presence of invasive and non-native species. The phylogenetic analysis revealed that *B. edithae* from Belitung Island formed a clade with *Betta* genera. The study concluded that the first record of DNA barcoding for *B. edithae* from Belitung Island and the subsequent genetic information obtained will contribute to future research on species identification, evolutionary relationships, genetic diversity, and population structure. These findings have important implications for conservation efforts and the management of fish populations, including the selection of prospective parents for domestication programs and determining the number of species populations based on genetic diversity.

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Conflict of interest. The authors declare no conflict of interest.

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